RESEARCH PAPER

Transporters involved in pH and K\(^+\) homeostasis affect pollen wall formation, male fertility, and embryo development

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Abstract

Flowering plant genomes encode multiple cation/H\(^+\) exchangers (CHXs) whose functions are largely unknown. AtCHX17, AtCHX18, and AtCHX19 are membrane transporters that modulate K\(^+\) and pH homeostasis and are localized in the dynamic endomembrane system. Loss of function reduced seed set, but the particular phase(s) of reproduction affected was not determined. Pollen tube growth and ovule targeting of \textit{chx17chx18chx19} mutant pollen appeared normal, but reciprocal cross experiments indicate a largely male defect. Although triple mutant pollen tubes reach ovules of a wild-type pistil and a synergid cell degenerated, half of those ovules were unfertilized or showed fertilization of the egg or central cell, but not both female gametes. Fertility could be partially compromised by impaired pollen tube and/or sperm function as CHX19 and CHX18 are expressed in the pollen tube and sperm cell, respectively. When fertilization was successful in self-pollinated mutants, early embryo formation was retarded compared with embryos from wild-type ovules receiving mutant pollen. Thus CHX17 and CHX18 proteins may promote embryo development possibly through the endosperm where these genes are expressed. The reticulate pattern of the pollen wall was disorganized in triple mutants, indicating perturbation of wall formation during male gametophyte development. As pH and cation homeostasis mediated by AtCHX17 affect membrane trafficking and cargo delivery, these results suggest that male fertility, sperm function, and embryo development are dependent on proper cargo sorting and secretion that remodel cell walls, plasma membranes, and extracellular factors.

Key words: Cation, endomembrane, male fertility, pollen, proton, transporter, wall formation.

Introduction

Early land plants, such as mosses and ferns, reproduce using motile male gametes that can swim through an aqueous medium to the female gametes. However, in flowering plants, two sperm cells are carried by a male gametophyte (pollen) to the egg cell and the central cell in the absence of an aqueous environment. After a pollen grain lands on a receptive stigma, it germinates and extends a pollen tube inside pistil tissues that surround the female gametophyte. Once inside
the embryo sac, the tube ruptures to release two sperm. One fuses with the egg cell and the other with the central cell to complete double fertilization (Dresselhaus and Franklin-Tong, 2013). Subsequent development of the embryo and the endosperm produces a desiccated seed that can remain viable for long periods of time. Although the major steps of reproduction have been known for decades, the molecular and cellular bases of pollen–pistil interactions that culminate in successful double fertilization are not well understood.

Recent studies have identified key molecular players in pollen–pistil sac interactions, including pollen tube attractants secreted by synergid (Okuda et al., 2009; Hitigashiya and Takeuchi, 2015) and egg cells (Márton et al., 2005); receptor-like kinases at the plasma membrane (PM) of pollen (Boisson-Dernier et al., 2009) and synergid cells (Escobar-Restrepo et al., 2007); and cell surface proteins of the sperm (HAP2/GCS1) (Mori et al., 2006; von Besser et al., 2006). Pollen germination and tip growth are accompanied by fluxes and oscillations of [Ca^{2+}], [K^+] or pH (Feijó et al., 2011). Their biological roles are mostly unknown. ACA9 is a pollen-specific Ca^{2+} influx or oscillations required for pollen germination and tip growth in vivo and aborted fertilization due in part to failed pollen tube rupture (Schiott et al., 2004). Thus disruption of Ca^{2+} efflux by ACA9 probably caused a defect in Ca^{2+} signaling, resulting in slowed tube growth, failed tube rupture, and reduced seed set. A single mutant of a cyclic nucleotide-gated ion channel, cngc18, showed male sterility due to a defect in pollen tube growth and inability to enter the transmitting tract (Frietsch et al., 2007). However, the single mutants cngc7 or cngc8 showed no defect, though pollen grains and tubes (Bock et al., 2006; Qin et al., 2009), though their biological roles are mostly unknown. ACA9 is a pollen-specific Ca^{2+} extrusion pump that is localized to the pollen tube PM. T-DNA insertional mutants show reduced pollen tube growth in vivo and aborted fertilization due in part to failed pollen tube rupture (Schiott et al., 2004). Thus disruption of Ca^{2+} efflux by ACA9 probably caused a defect in Ca^{2+} signaling, resulting in slowed tube growth, failed tube rupture, and reduced seed set. A single mutant of a cyclic nucleotide-gated ion channel, cngc18, showed male sterility due to a defect in pollen tube growth and inability to enter the transmitting tract (Frietsch et al., 2007). However, the single mutants cngc7 or cngc8 showed no defect, though pollen grains and the double mutants were sterile, possibly due to a defect in pollen tube growth and inability to enter the transmitting tract (Frietsch et al., 2007). Curiously, quadruple mutants (chx17chx18chx19) were recovered at a lower frequency than expected. Furthermore, chx17chx18chx19 recovered growth on low K^+ medium and mediated 86Rb^+ uptake. Furthermore, CHX17 as well as CHX18 and CHX19 restored growth of yeast lacking K^+ uptake transporters (Chanroj et al., 2011). These results in yeast are consistent with their role as a K^+/H^+ antiporter at the endomembrane; though the mode of transport at the PM is less clear (Chanroj et al., 2011; Mottaleb et al., 2013). Structural modeling predicts that AtCHX17 protein has an NhaA-fold architecture, and mutagenesis showed core residues at positions similar to cation/H^+ exchangers (Czerny et al., 2016).

The roles of CHX17, CHX18, and CHX19 in planta are less clear. CHX17 is expressed in roots, though vegetative growth of seedlings in single, double, or triple mutants (chx17chx18chx19) under various stress conditions was unaltered (Chanroj, 2011). Curiously, quadruple mutants (chx16chx17chx18chx19) were recovered at a lower frequency than expected. Furthermore, their quadruple mutant pod contained 60% fewer seeds than the wild type (Chanroj et al., 2013). Homozygous triple mutants, chx17chx18chx19, were also recovered at a lower than expected frequency. Here, we determine which phase of reproduction led to reduced seed set in mutants. We demonstrate that chx17chx18chx19 pollen tubes grow, target, and enter ovules; however, many targeted ovules fail to develop into seeds, suggesting a failure to complete fertilization. Our findings underscore a previously unknown role for endomembrane transporters and K^+ and pH homeostasis in male fertility, fertilization, and embryo development possibly through remodeling of the cell wall and PM.

Materials and methods

Plants and genotyping

Arabidopsis thaliana, Columbia-0, was grown in Miracle-Gro® potting mix supplemented with 5% Perlite. Plants were grown under a 16 h photoperiod at 150 μE m^{-2} s^{-1} illumination, 21 °C, and 60%
relative humidity. All chx mutants used (see Supplementary Table S1 at JXB online) are available from ABRC.

Genotype was determined by PCR using two gene-specific primers or one gene-specific primer and a T-DNA border primer (Supplementary Table S2). Genomic DNA was isolated from 1-2 rosette leaves of ~5-week-old plants (Edwards et al., 1991). DNA was amplified by Taq DNA polymerase (NEB M0273L) in a three-step PCR program for 40 cycles. PCR products were separated on a 1% agarose gel and visualized using ethidium bromide.

Segregation analysis

To test male transmission of the mutant allele, pollen from a parent carrying a heterozygous gene was transferred to a pistil carrying the wild-type gene. To test female transmission, a stigma heterozygous for a gene was given pollen harboring the wild-type gene. Parental plants were genotyped for wild-type genomic sequence or T-DNA insertions in CHX17, CHX18, and CHX19 genes. Stage 12 flower buds (Smyth et al., 1990) were emasculated, and pistils were pollinated 2 d later, with anther from stage 13 flowers. Seeds developed on the plant for 10-12 d until pods dried. F1 seeds were planted, and genomic DNA from ~100 plants was extracted for genotype analyses.

Analysis of wild-type and chx17/18/19 plants

Silique samples from the primary bolts of 9-week-old plants were collected starting from the fourth pod below the inflorescence. Pod lengths were measured in ImageJ (NIH) using at least 10 plants per line. Four pods of average length per plant were split open and seeds were scored under a stereomicroscope (Nikon SMZ1000).

To detect nuclei, pollen grains from 1-3 stage 13 flowers (Smyth et al., 1990) were dabbed onto a microscope slide, and incubated with 30 µl of DAPI solution for 15 min. DAPI (Life Technologies D3571) solution contained 0.1 M sodium phosphate pH 7, 1 mM EDTA, 0.1% Triton X-100, and 0.4 µg ml1 DAPI. Pollen was examined by Nikon E600 with a UV filter (excitation/emission at 360/410 nm).

To visualize pollen tubes in vivo, pollinated pistils were stained with aniline blue to label callose (Mori et al., 2006). Self-fertilized wild-type and chx17/18/19 pistils were analyzed 1-4 days after pollination. DAPI (Smyth et al., 1990). Pistils were fixed in 75% ethanol/25% acetic acid for 2 h, and then rehydrated in 75, 50, and 30% ethanol, and deionized water. The pistils were incubated in 8 M NaOH overnight, washed once with deionized water, and then incubated for 2 h in 0.1% decolorized aniline blue (Fisher Scientific A-967) in 100 mM K2HPO4 at pH 10. Each pistil was placed on a glass slide and pressure was applied to expose ovules and the transmitting tract. Images were recorded using a Nikon E600 fluorescence microscope with a UV filter (excitation/emission wavelengths at 360 nm/410 nm).

Ovule development from self- or manually pollinated pistils was visualized using chloral hydrate. Seed pods were fixed in 90% ethanol/10% acetic acid overnight at room temperature. Pods were washed twice for 30 min in 90% ethanol and then cleared in chloral hydrate/glycerol/water (8:1:2) solution (Berleth and Jurgens, 1993). Each pod was opened and all ovules or developing seeds were mounted in chloral hydrate solution and examined with DIC (differential interference contrast) microscopy.

To examine pollen wall architecture, pollen grains from stage 13 flowers were mounted on stubs over double-sided tape. The specimens were then sputter coated with gold–palladium (60%:40%) (Balzers Med 010) and observed using a scanning electron microscope (SU3500, Hitachi) at an accelerating voltage of 3 kV in high vacuum.

Monitoring synergid degeneration and gamete fusion

To visualize sperm nuclei, HTR10: HTR10:RFP was introduced into the chx17/chx18/chx19 mutant by genetic crossing. Self-fertilized chx triple mutants with red fluorescent protein (RFP)-labeled sperm were recovered. Oddly, plants showed a range of RFP-labeled pollen, ~20-50%, and a few plants with >55% RFP. A triple chx mutant containing 73% RFP-labeled pollen was selfed, and progeny yielded flowers in which 85% of the pollen was RFP positive.

Stage 12b or 12c buds (Smyth et al., 1990) were emasculated and hand pollinated 24 h later. Hand pollinations were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating emasculated ACT11:MSI1:GFP stigmas with pollen from male donor plants carrying HTR10: HTR10:RFP (Ingoff et al., 2007). Pollinated pistils were analyzed 16 h after manual pollinations. Pistils were harvested and ovary walls were removed as previously described (Johnson and Kost, 2010). Dissected pistil tissues were mounted in 80 mM sorbitol for analysis by confocal laser scanning microscopy (CLSM) using a x40 objective with DIC capability (Zeiss LSM800 upright microscope). Green fluorescent protein (GFP) expression was imaged using a diode laser at 10 mW with excitation at 488 nm and emission at 509 nm. mRFP (modified RFP) expression was imaged with a diode laser at 10 mW with excitation at 633 nm and emission at 670 nm. Signal intensities were optimized for each fluorophore and then combined in overlay.

Final images represent a merge of single planes at varying depths (z stacks). Synergid status was determined based upon the visualization of nuclear ACT11:MSI1:GFP signal as described before (Leydon et al., 2015).

Accession numbers

CHX17 (At4g23700), CHX18 (At5g41610), and CHX19 (At3g17630)

Results

chx17/chx18/chx19 mutants show reduced seed set

Vegetative growth and flower development of chx17/chx- 18/chx19 plants were similar to those of the wild type (Fig. 1A-i; Supplementary Fig. S1). However, seed pods of chx17/chx18/chx19 plants were shorter, varying from 8.8 mm to 10.3 mm, compared with 13.0 mm in the wild type (Fig. 1A-ii, B; Supplementary Fig. S2). When siliques were cleared, mutant pods had random gaps instead of two continuous rows of seeds in wild-type pods (Fig. 1A-iii, iv). Progeny from three chx17/chx18/chx19 plants showed, on average, 11-21 seeds, compared with 48 seeds per pod in the wild type (Fig. 1C). Seed set or pod length of double mutants chx17/chx18 or chx17/chx19 were similar to those of the wild type (Fig. 1B, C). Thus, loss of function of three CHX genes, CHX17, CHX18, and CHX19, reduced seed set to half that seen in the wild type.

Male fertility is compromised in chx17/chx18/chx19 pollen

Segregation distortion in the progeny from a selfed mutant carrying heterozygous CHX18+/– in a double chx17/chx19 mutant background (Chanroj et al., 2013) indicated a gametophytic defect. Reciprocal crosses were conducted to determine whether transmission of T-DNA-disrupted genes was due to the male or the female gametophyte, or both. As double mutants behaved like the wild type, we first tested segregation of a heterozygous CHX18+/– or CHX19+/– gene in a double mutant background crossed to the same double mutant. For instance, pollen from a double chx17+/–chx19+/– mutant plant
carrying a heterozygous $CHX18^{+/−}$ gene was placed onto the same double mutant pistil carrying $CHX18^{+/+}$ (Table 1). Transmission of $chx18-1$ and $chx19-2$ mutant alleles was tested by genotyping the $F_1$ progeny. Parents were genotyped for mutant or wild-type alleles of $CHX17$, $CHX18$, and $CHX19$ by PCR prior to crossing (Fig. 2A, B). The $F_1$ progeny were tested for homozygous wild-type $CHX18^{+/+}$ or heterozygous $CHX18^{+/−}$. PCR-amplified products of 1 kb or 0.5 kb were used to distinguish either the wild-type or mutant allele, respectively (Fig. 2C). If a $CHX$ gene has minimal or no effect in male fertility, then progeny containing homozygous wild-type $CHX18^{+/+}$ would be approximately equal to progeny carrying heterozygous $CHX18^{+/−}$.

Transmission of the $chx18-1$ allele through pollen of the $chx17chx19$ double mutant background was 5% instead of the 50% expected (Table 1). Similarly, transmission of the $chx19-2$ allele through pollen in a $chx17chx18$ mutant background was only 3% instead of 50% (Table 1). In contrast, the average transmission of the $chx18-1$ allele through the female was 30% instead of 50% (Table 1). Similarly, transmission of the $chx19-2$ allele was 25% instead of 50% (Table 1). Together, these results indicate that $chx17chx18chx19$ gametophytes are significantly less fertile than either $chx17CHX18chx19$ or $chx17chx18CHX19$ double mutants. Thus one functional $CHX$ out of three is required for either male or female fertility. We conducted additional reciprocal crosses with the wild type in order to define the impact on male and female reproductive function. When pollen from a double $chx17chx19$ mutant that is heterozygous for $CHX18^{+/−}$ was transferred to a wild-type pistil, the fertility of the triple $chx$ mutant pollen was severely reduced, as only 10% of the $F_1$ progeny carried a triple heterozygous genotype (Table 2), a significant deviation from the expected 50%. In the reciprocal cross using wild-type pollen, female transmission of the triple $chx$ mutant was 38% instead of 50%, consistent with a role for $CHX$ genes in the female gametophyte (Table 2). These data further support our hypothesis that at least one copy of $CHX17$, $CHX18$, or $CHX19$ is required for complete male and female gametophyte function. Since the defect in male reproductive function was more pronounced, we focused on defining the roles of $CHX17$, $CHX18$, and $CHX19$ in the male gametophyte.

**Fig. 1.** Triple $chx17chx18chx19$ mutant plants showed reduced seed set. (A) Plants and seed pods. (i) Wild-type (WT) Columbia-0 and $chx17/18/19$ plants have similar vegetative and reproductive growth. (ii) $chx17/18/19$ mutant pods were shorter than those of the WT. (iii) $chx17/18/19$ pods contained fewer seeds. Scale bar=1.0 mm. (iv) $chx17/18/19$ pods contained aborted and developing seeds. At least 44 pods per genotype were analyzed. Scale bar=0.5 mm. (B) Pod lengths of the $chx17/18/19$ mutant are 21–29% shorter than pods of the WT or $chx17/18$ or $chx17/19$ double mutants. Results show the mean, and bars represent the SE ($n$=234–610 pods). (C) Seed number per pod was reduced 55–73% in the $chx17/18/19$ mutant relative to the WT or the $chx17/18$ and $chx17/19$ double mutants. Four siliques of average length were split open per plant to count seeds. Results show the mean, and bars represent the SE ($n$=44–126 pods).

$chx17chx18chx19$ mutant pollen contains three nuclei though the wall pattern is disorganized

The size and morphology of mutant and wild-type pollen grains from stage 13–14 flowers appeared similar under light microscopy. Pollen grains were stained with DAPI which binds dsDNA. Fluorescence microscopy showed that 95% of triple mutant grains contained three DAPI-stained nuclei like the wild type (Fig. 3A). About 5% of mutant grains showed
Pollen grains from a double mutant chx17chx18 parent heterozygous for CHX18+/– were manually transferred to a double mutant pistil carrying wild-type CHX18+/+ (a). Reciprocal crosses were also conducted (b). Similar reciprocal crosses were performed with the double mutant chx17chx18 heterozygous for CHX19+/– (c) and the double mutant alone. Seeds from these crosses were planted and leaves were collected for DNA extraction and genotyped. The observed (Obs) segregation of F1 progeny is shown below as the number of individuals recovered or percentage (%).

<table>
<thead>
<tr>
<th>Female: egg or central cell</th>
<th>Male: pollen or sperm</th>
<th>F1 progeny</th>
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<td>50 62 (95)</td>
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<tr>
<td>b – – + – –</td>
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<td>50 3 (5)</td>
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<td>c – – – + –</td>
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<td>d – – – – +</td>
<td>– – – +</td>
<td>–/– –/– +/–</td>
<td>50 41 (30)</td>
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Table 1. Only one of three CHX genes is sufficient to restore male fertility

Intriguingly, SEM showed that the outer wall or exine of chx17chx18chx19 mutant pollen grains was disorganized, in contrast to the highly reticulate pattern of wild-type grains (Fig. 3C). The single chx17 mutant pollen displayed wall sculpture resembling that of wild-type grains (Supplementary Fig. S3). However, the double chx17chx18 mutant grains gave a mixture of wall patterns where ~52% were fully disorganized, 16% were intermediate, and 31% looked normal (Supplementary Fig. S3). Thus all three CHX genes have overlapping roles in pollen wall formation and patterning.

chx17chx18chx19 pollen tubes arrive at ovules, but seed development fails

Pollen tube growth in the pistil was examined using aniline blue. Callose from vascular tissue of the funiculus is distinct as seen in unpollinated pistils. At 1 DAP, wild-type pollen tubes are visible at the base of the pistil (Supplementary Fig. S4). Furthermore, most ovules (74–82%) had received a pollen tube, observed at higher magnification (not shown). The estimate is probably low as many tubes were not visible due to ovule crowding. Two days after wild-type pollination, 93% of seeds (568 out of 611) had enlarged, though pollen tubes were mostly undetectable, suggesting that successful fertilization caused degeneration of pollen tubes. In wild-type pistils pollinated with the triple mutant, many tubes were visible along the length of the pistil at 1 DAP, though fewer tubes were visible at the base, suggesting that pollen germination, tube growth, or both are suppressed. By 2 DAP, some ovules have increased in size, consistent with early seed development after successful fertilization (Supplementary Fig. S2). However a significant number of ovules (250%) remained small and are referred to as ‘undeveloped ovules’. At 3 DAP, developing ovules/seed increased in size further; however, the ‘undeveloped ovules’ from triple mutant pollen had begun to shrivel (Supplementary Fig. S4). At 2–3 DAP, we noticed that aniline blue-stained pollen tubes were less or not visible in developing ovules, confirming that the pollen tube degenerated in cases when fertilization is successful.

To distinguish between size differences of undeveloped and developed ovules in pistils pollinated with triple mutant pollen, we analyzed mutants at 2 DAP. We could not see all undeveloped ovules as some are obstructed by the transmitting tract, so we only scored pollen tubes in any undeveloped ovules in full view. For example, we counted 50 pollen tubes inside 132 undeveloped ovules, which included 28–57% pollen reception by undeveloped ovules per pistil. Thus, on average, at least 39% of the undeveloped ovules had received a tube, while a fraction of undeveloped ovules did not receive a tube (Supplementary Fig. S5). In contrast, pistils pollinated with wild-type pollen show that most ovules (86–97%) have increased in size by 2 DAP (Supplementary Fig. S4). Thus mutant pollen grains germinate, extend tubes, and are able to target ovules; however, tube number and tube lengths are reduced relative to the wild type. Even though mutant pollen tube lengths grown in vitro for 6 h were 10–20% shorter and the percentage germination was less (14–32%) than in the wild type (58%) (data not shown), on average most (65–80%) ovules received a tube when pollen grains germinated in vivo on the stigma.

We found that growth of mutant pollen tubes in self-pollinated triple mutants is slightly more robust than that manually transferred to wild-type pistils. In pods of self-pollinated wild-type plants, fluorescent-labeled pollen tubes were visible outside each developing ovule/seed (not shown). Mutant chx17chx18chx19 pollen tubes also grew through the female transmitting tissues to the distal end of the pod, as evident from developing seeds (Fig. 4A). Though triple mutant tubes did reach the distal end, many small undeveloped ovules were seen along the entire length of the pod. Upon closer examination, a pod at the right developmental stage (2–3 DAP)
showed that 15 out of 21 undeveloped ovules had received a tube (Fig. 4A). Some undeveloped ovules at the distal end of the pod were homogeneously gray and did not receive a tube (Fig. 4A, far right) thus they were unfertilized. Other undeveloped ovules with a visible pollen tube showed bright grainy content (Fig. 4C, right), suggesting abnormal development after tube reception. Out of three pods at a similar developmental stage, we counted 41 pollen tubes entering 65 undeveloped ovules, indicating that at least 63% of undeveloped ovules have received a tube. In three separate experiments using 10 pods each, we detected that pollen tubes had entered 30–63% of the undeveloped ovules in the pistils from the triple mutant (Fig. 4B, C). These results indicated that the pollen–pistil interactions regulating in vivo pollen tube growth and tube guidance of the triple mutant were not perturbed. Mutant tubes are able to sense chemical cues in the pistil and those from the embryo sac and respond by targeting the micropyle. The results indicate that even though pollen tubes reach ovules, there is a failure to complete fertilization.

Some undeveloped ovules at the distal end of the pod were homogeneously gray and did not receive a tube (Fig. 4A, far right) thus they were unfertilized. Other undeveloped ovules with a visible pollen tube showed bright grainy content (Fig. 4C, right), suggesting abnormal development after tube reception. Out of three pods at a similar developmental stage, we counted 41 pollen tubes entering 65 undeveloped ovules, indicating that at least 63% of undeveloped ovules have received a tube. In three separate experiments using 10 pods each, we detected that pollen tubes had entered 30–63% of the undeveloped ovules in the pistils from the triple mutant (Fig. 4B, C). These results indicated that the pollen–pistil interactions regulating in vivo pollen tube growth and tube guidance of the triple mutant were not perturbed. Mutant tubes are able to sense chemical cues in the pistil and those from the embryo sac and respond by targeting the micropyle. The results indicate that even though pollen tubes reach ovules, there is a failure to complete fertilization.
Table 2. Reciprocal crosses show that male fertility is compromised in the chx17chx18chx19 mutant

Pollen grains from a double mutant chx17chx19 parent heterozygous for CHX18 were used to pollinate a wild-type stigma (a). Wild-type pollen grains were used to pollinate a double mutant pistil heterozygous for CHX18−/− (b). F1 seeds from these crosses were planted and leaves were collected for DNA extraction and genotyped. The observed (Obs) segregation of F1 progeny is shown below as number of individuals recovered or percentage (%). Exp. refers to the expected ratio of F1 progeny in %.

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<td>a  +  +  +</td>
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<td>b  –  +  –</td>
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The number of F1 plants tested was 112–115. χ² >6.64 (P value of 0.01) indicates that the observed (Obs) results are significantly different from the expected (Exp) Mendelian ratio.

![Fig. 3. Mutant chx17chx18chx19 pollen grain contains three nuclei and a disorganized wall pattern. (A) Representative images of DAPI-stained pollen grains observed by UV fluorescence (top) or bright-field (bottom) microscopy. Scale bar=10 µm. (B) Nearly all wild-type and chx17/18/19 pollen grains contained three nuclei as visualized by DAPI staining. Total grains scored were 108 and 200 from the wild type and chx17/18/19, respectively. (C) Loss of reticulate wall pattern. Scanning electron micrographs of the wild type (Wt; i, ii) and chx17/18/19 mutant pollen grains (iii, iv). Scale bar=10 (i, iii) or 5 µm (ii, iv).](image)

Embryo and endosperm (30%) (Fig. 5F). Pods from the control wild type yielded nearly all normal seeds (98%), similar to pods obtained from triple mutant pistils pollinated by wild-type pollen (98%) (Fig. 5F). These results are consistent with the reciprocal cross study where male fertility is severely compromised by loss of function of three CHX genes in the male gametophyte.

Pods of self-pollinated triple chx17chx18chx19 mutants also contained three classes of ovules: (i) 33% unfertilized ovules; (ii) 11% single-fertilized ovules; and (iii) 55% normal developing seeds (Fig. 5A–E). The percentage of undeveloped ovules that includes unfertilized and single fertilized ovules ranged from 45% to 70% based on analysis of six pods. Unfertilized ovules were verified by the presence of a central nucleus or egg cell, as shown in Fig. 5B. Products of single fertilization, though less frequent, were consistently observed mostly as a globular embryo only (Fig. 5C). We also noticed developing endosperm without any visible embryo (Fig 5D). These results indicate that only one fertilization event was successful. Ovules with endosperm alone varied in size, and can increase to twice the length of the unfertilized ovule.
About one-third to half of the ovules per pod developed normally, judging by the development of both an embryo and an endosperm, indicating that a subset of triple mutant pollen was competent to complete double fertilization with triple mutant female gametes (Fig. 5E). It is clear that ovules are targeted by chx triple mutant pollen tubes (Fig. 4), yet fail to develop into seeds (Fig. 5). However, given the expression pattern (Fig. 5G) of CHX17, CHX18, and CHX19, this could be due to a defect in interactions between mutant pollen tubes and female gametophytes, or to failed interactions between mutant sperm and female gametes. To resolve the spatial and temporal relationship of pollen tube interactions with the embryo sac and the success of sperm fusion with the female gametes, we used an assay that simultaneously monitors synergid degeneration and the status of sperm cells (Leydon et al., 2015). After a pollen tube enters and contacts the ovule, one of two synergid cells degenerates. Plants carrying ACT11:MSII:GFP express nuclear GFP in all cells, but the synergid cells express it most strongly (Fig. 6B). As the receptive synergid degenerates, the nuclear GFP deteriorates and loss of the nuclear integrity is especially clear when the GFP signal diffuses throughout the synergid cytoplasm (Fig. 6B). After wild-type ACT11:MSII:GFP pistils were pollinated with wild-type pollen carrying HTR10:HTR10:RFP, which labels sperm nuclei (Ingouff et al., 2007), 81% of ovules showed either one or no intact synergid nuclei, and diffuse HTR10:RFP signal overlapping the egg cell nucleus and central cell nucleus, indicative of successful pollen tube reception and double fertilization (Fig. 6A–C, J–K). The remaining 19% of ovules had no visible sperm cells, though 334 out of 443 ovules showed that at least one synergid had undergone degeneration (Fig. 6D–F, J, K). The degeneration of the synergid cell nuclei indicates that mutant pollen tube had reached the synergid cell, consistent with the observation of sperm cells localized to the micropylar region of the ovule (Fig. 6G). Unfused sperm cells were observed in 3.2%
(or 14) of the ovules (Fig. 6H, I); these ovules showed synergid degeneration with no sign of plasmogamy; in many cases, the sperm appeared to be deposited incorrectly at the micropylar end of the synergid, rather than at the interface between the egg and the central cell where gamete fusion takes place (Hamamura et al., 2011). Only 1.6% (seven) ovules showed successful double fertilization and had either one or no intact synergids (Fig. 6J, K). Thus the frequency of double fertilization was extremely low in chx triple mutant pollen, though the frequency of synergid degeneration is high. Thus chx triple mutant pollen tubes can target ovules and undergo normal pollen tube reception as evidenced by synergid degeneration.

Fig. 6. Live imaging demonstrates that chx17chx18chx19 pollen tubes trigger synergid degeneration, yet sperm frequently fail to undergo fertilization. (A–I) Confocal micrographs of ACT11:MSI1:GFP ovules receiving pollen tubes expressing a sperm-specific HTR10:HTR10:RFP, 16 h after pollination. Scale bars=20 μm. (A–C) Wild-type HTR10:HTR10:RFP sperm cells are visible in ovules with a diffuse signal (A) (arrows) that overlaps with the nuclear GFP signal (B) from the egg and central cell indicating the successful fusion of gametes and formation of the zygote nucleus (zn) and endosperm nucleus (en). The synergid nucleus of the receptive synergid has degenerated (dsn) and the non-receptive synergid nucleus is still intact (sn), indicating that pollen tube reception has taken place normally. (C) Overlapping RFP and GFP signal. (D–I) Ovules receiving chx17chx18chx19, HTR10:HTR10:RFP sperm cells frequently lack positive signal from the HTR10:HTR10:RFP reporter. (D) Sperm cell signal of HTR10:HTR10:RFP is visible in pollen grains that have detached from the stigma into the media (pollen grains, top left), yet not inside ovules. (E) Ovules show evidence of pollen tube reception and synergid degeneration by the degeneration of one or both synergid nuclei (dsn). (F) Overlapping GFP and RFP signals indicate a loss of visible RFP signal, yet the egg (ecn) and central cell nuclear (ccn) GFP signal remains strong. (G) Overlapping RFP and GFP signal from an ovule with two sperm cells (sc) approaching the micropyle, indicating normal pollen tube arrival at the ovule prior to reception. Ovules with one (H) or two (I) degenerated synergid nuclear GFP signals (dsn) and one visible HTR10:HTR10:RFP-positive sperm cell that has failed to undergo fusion with either the egg or the central cell. (J) Quantification of the HTR10:HTR10:RFP sperm signal in all ovules receiving wild-type or chx17, 18, 19 pollen tubes. (K) Quantification of nuclear ACT11:MSI1:GFP signal in synergids in ovules from (J), 0 SN, zero intact synergid nuclei; 1 SN, one intact synergid nucleus; 2 SN, two intact synergid nuclei. The total number of ovules observed for each category is written above each stacked column, and the number of ovules in each synergid status category is written on each stacked column unit.
Therefore, reduced male fertility of chx17chx18chx19 triple mutants is due to partial failure in pollen discharge, sperm positioning, impaired sperm–egg interactions, sperm degeneration during pollen tube growth, or a combination of these defects.

**Delayed embryo development of self-pollinated triple mutants**

Most embryos within a wild-type silique develop at similar rates, so they reach the late heart stage by 5 DAP (Fig. 7A). Similarly, most of the embryos (80–90%) produced from a wild-type pistil pollinated with triple mutant pollen developed to the late heart stage by 4–5 DAP (Fig. 7G). However, when fertilization was successful in self-pollinated triple mutants, embryos within a silique showed a range of developmental stages (Fig. 7B–E). When some embryos had reached the late torpedo stage, others were at the globular or early to late heart stages (Fig. 7H). Thus, homozygous triple mutant embryos showed delayed development. From six pods, we estimated that ~55% of the embryos had not reached the heart stage, when 30% were at the early to torpedo stage. The fate of embryos with severely delayed development was not followed so it is unclear whether development would abort prematurely, or progress to full maturity. These results suggest that CHX functions are also critical for post-fertilization development of the young sporophyte. As a wild-type pistil pollinated with triple mutant pollen produced embryos that developed similarly to the wild type, our results suggest that at least one copy of CHX17, CHX18, or CHX19 is required for synchronous development of mutant embryos.

**Fig. 7.** The self-pollinated chx17chx18chx19 mutant also show delayed embryo development when fertilization is successful. Six days after pollination, pods were fixed and cleared with chloral hydrate. (A) Wild-type (WT) embryos are mostly at the torpedo stage. (B–F) Mutant ovules at various developmental stages from a single pod: (B) globular, (C) early heart, (D) late heart, (E) early torpedo, and (F) torpedo. Scale bars=100 μm. (G) The embryo developed to a similar stage when a wild-type pistil received chx17/18/19 mutant pollen or vice versa. The percentage of embryo stages per pod from (i) WT×WT cross; (ii) WT pistil×mutant pollen; or (iii) mutant pistil×WT pollen. (H) Self-pollinated triple mutants showed delayed embryo development. WT or triple chx mutants were self-pollinated and pods were examined by DIC microscopy. Results are the average of 10 pods per treatment.
Discussion

We take advantage of a *chx17chx18chx19* mutant to dissect the basis of reduced seed set and shed light on the specific reproductive phase(s) affecting male fertility. Reduced male fertility is attributed to multiple steps, including germination, tube growth, pollen discharge, and fertilization defect in sperm. Our studies highlight the importance of endomembrane transporters involved in pH and cation homeostasis. Combined with previous functional studies, these results suggest that CHX transporters influence reproductive development through membrane trafficking by possibly remodeling cell walls and the PM.

Male transmission defect and failed fertilization

Based on reciprocal crosses and the low transmission of the mutant *chx18* or *chx19* allele (3–5% instead of 50%) by the male gametophyte to the F1 progeny, we concluded that a male gametophyte with loss of function in *CHX17*, *CHX18*, and *CHX19* genes was severely impaired. In contrast, transmission of the mutant *chx18* or *chx19* allele by the female gametophyte was 25–30% instead of 50%, thus a triple mutant female gametophyte was less severely compromised.

The basis of the male gametophyte defect was apparently not due to development of the microspore into the mature pollen based on light microscopy. The male gametophyte begins after meiosis of a pollen mother cell in the anther, forming four haploid microspores. Each microspore divides to produce a bicellular pollen containing a vegetative cell and a generative cell. The generative cell then undergoes mitosis to produce two sperms cells, thus giving rise to a tricellular mature pollen. DAPI staining showed that 95% of triple mutant grains contained three nuclei, similar to those of wild-type grains, indicating that male gametogenesis was largely unaffected by loss of *CHX17*, *CHX18*, and *CHX19* activity. Ultrastructural studies confirmed that the mutant developed like the wild type (not shown); however, SEM revealed a disorganized wall in mutant pollen.

Mutant pollen had a ‘spongy’ wall instead of a reticulate pattern, though that defect alone is unlikely to reduce male fertility (Dobritsa et al., 2011). The outer wall of pollen grains protects against dessication, and enhances adhesion and hydration on the stigma before pollen germination. Germination of *chx17chx18chx19* pollen was reduced and tube lengths were shorter *in vitro*; yet the quantity of pollen *in vivo* ensured that sufficient grains attached to the stigma and many tubes grew into the ovary. Thus pollen functions in the early phases of reproduction were not severely hampered. Aniline blue staining demonstrated that mutant pollen tubes had targeted and entered many ovules, indicating that loss of *CHX17*, *CHX18*, and *CHX19* function did not affect the ability of tubes to sense cues secreted by the female gametophyte and to target the embryo sac (Higashiyama and Takeuchi, 2015).

Pollen tubes were observed in both developing and undeveloped seeds, suggesting that the pollen tube had entered the micropyte in both cases. About 39–65% of undeveloped ovules in each pod had received a pollen tube, indicating that fertilization was unsuccessful. Moreover, mutant pollen tube reception was verified by the degeneration of one or both synergid cells seen in 80% ovules of a wild-type pistil (Fig. 6K). In wild-type pollen, both sperm cells are released simultaneously at tube rupture after synergid cell reception (Hamamura et al., 2011). One sperm fuses with the egg cell to form a zygote that develops into the embryo. The second sperm nucleus unites with the central cell nucleus and undergoes subsequent nuclear division and cellularization to produce the endosperm. Direct analysis of *chx* triple mutant sperm cells in female gametophytes suggests that many pollen tubes may fail to deliver sperm. The vast majority of ovules pollinated with triple mutant pollen had no sperm cells visible based on RFP fluorescence (Fig. 6K). Failure to express markers of sperm identity such as HTR10::HTR10::RFP (Fig. 6) may indicate that mutant sperm cells are compromised. The lack of visible sperm signal could account for the apparent low frequency of successful fertilization estimated from the imaging results (Fig. 6) compared with 27–45% of developing seeds per pod in pistils receiving *chx* mutant pollen *in vivo* (Fig. 4).

Undeveloped ovules that received a pollen tube suggest that either tube rupture was defective, or released sperm cells fail to complete fertilization with the female gametes, or both. Single fertilization events were observed, indicating that defective tube rupture is not the major cause. Some ovules develop only a globular embryo with no detectable endosperm tissue, and other ovules enlarge in size due to proliferation of the endosperm nuclei, with no visible embryo. Either of these examples will lead to abortion and failed seed development, as embryogenesis and endosperm development are co-ordinated to produce successful seed development (Berger, 2003). This idea is verified by the presence of unfused sperm cell in ovules pollinated by *chx17chx18chx19* mutant pollen (Fig. 6H, I, K). Single fertilization events suggest that one of the two sperm failed to complete fertilization. Thus loss of *CHX17*, *CHX18*, and *CHX19* genes compromised sperm function in half to two-thirds of the male gametophytes, leading to failed double fertilization.

The ability of any one of three *CHX* genes to rescue male fertility is unexpected as they are differentially expressed in space and time. *CHX17* transcript detected in microspore and bicellular pollen (Honys and Twell, 2004) was confirmed by CHX17::GUS (β-glucuronidase) staining of anthers in stage 9–10 flowers (Bock et al., 2006). *CHX19* transcript is detected in developing pollen grains and tubes (Fig. 5G), where the protein was localized to the PM (Supplementary Fig. S6). *CHX18* transcript was detected in sperm cells (Fig. 5G) isolated by fluorescence-activated cell sorting (Borges et al., 2008), whereas *CHX19* expression is low or ‘absent’. In contrast, *CHX18* transcript was low or not detectable in mature pollen (Honys and Twell, 2004) or in the tube (Qin et al., 2009), suggesting that *CHX18* transcript in sperm cells is not contaminated by mRNA from the vegetative cell. *HAP2/GCS1* or *GEX2* are experimentally demonstrated to be sperm-expressed genes (Mori et al., 2006, 2014; von Besser et al., 2006), whereas Feronia is not expressed in pollen and
is expressed in synergid cells of the female gametophyte (Huck et al., 2003; Rotman et al., 2003; Escobar-Restrepo et al., 2007). The transcriptome results of reproductive tissues have been verified by these studies (Fig. 5G), indicating that AtCHX18 and AtCHX17 are expressed in sperm cells. At least one of three CHXs is expressed at every stage of male gametophyte development. Apparently one copy of CHX19 is sufficient to restore male fertility of a chx17chx18 double mutant. Thus plants have mechanisms to compensate when one or more homologous genes are rendered non-functional.

Yet some chx17chx18chx19 mutants completed double fertilization successfully. This partial success in fertilization by triple mutant sperm could be due to compensation by other sperm-expressed cation/H+ exchangers, such as AtCHX20 (see Fig. 5G) or AtNHX2. AtCHX20 also mediates cation and pH homeostasis in yeast (Padmanaban et al., 2007), though it differs from CHX17, in endomembrane localization and inability to sort cargo, such as aminoglycoside HygB, in yeast (Chanroj et al., 2011).

The female gametophyte and post-fertilization events contribute to seed development

The basis for transmission of the CHX18+ allele via the female gametophyte (Table 2) awaits further investigation. Both CHX17 and CHX18 transcripts were detected in the central cell (Schmid et al., 2012) (Supplementary Fig. S7), and possibly in the synergid and egg cell (Wuest et al., 2010; S. Sprunck, personal communication).

We showed that one functional CHX17, CHX18, or CHX19 is also critical for post-fertilization development, as early embryo development contained in one pod was synchronous in a heterozygous chx17+/−chx18+/−chx19+/− sporophyte but was delayed in a homozygous chx17+−chx18+−chx19+− mutant (Fig. 7G, H). We suggest that after fertilization, early embryo development depends on a functional CHX17 or CHX18 in the embryo, endosperm, or both. Several observations support this idea. First, CHX17p::GFP expression is strong at the anterior end of the developing seed (Chanroj et al., 2013) consistent with CHX17 expression at the micropylar endosperm of the early embryo (Belmonte et al., 2013). Secondly, CHX18 is highly expressed in the micropylar and the peripheral endosperm at the pre-globular and globular embryo stage according to the transcriptome of Arabidopsis seed development (Supplementary Fig. S8) (Belmonte et al., 2013). Thirdly, RNA sequencing revealed CHX17 and CHX18 transcripts in a laser-dissected central cell (Supplementary Fig. S7) (Schmid et al., 2012). Union of a sperm cell with the central cell nucleus and subsequent proliferation produces a micropylar endosperm that surrounds the embryo. The role of the micropylar endosperm is unclear. A recent study demonstrated that cysteine-rich peptides, ESF1 (Embryo Surrounding Factor) accumulated in the central cell before fertilization and in the micropylar endosperm after fertilization (Costa et al., 2014). Furthermore, purified ESF has a role in early development of the embryo, possibly through suspensor elongation and auxin distribution. These results suggest that the endosperm surrounding the embryo plays a critical role in producing peptide cues to facilitate development of the suspensor and pre-globular embryo. One model is that CHX17 and CHX18 influence peptide sorting and secretion from the micropylar endosperm to the target cells.

Model: CHX17, CHX18, and CHX19 activities affect PM and wall remodeling

How would loss of CHX17, CHX18, or CHX19 produce the various defects we observed? The loss in exine patterning of chx17chx18chx19 mutant grains may be indirectly related to decreased male fertility, yet it may provide a clue to compromised wall formation. There are two schools of thought regarding outer wall formation: (i) primexine (precursor of exine) and exine seen on microspores depend on materials synthesized from tapetum (or sporophyte); and (ii) primexine and exine are formed by the combined activities of the microspore and tapetum (Dobritsa et al., 2011). Ultrastructural studies had suggested that primexine originated from the PM of the microspore. For instance, the dex1 mutant is male-sterile, and a tetrad of dex1 microspores surrounded by callose wall shows defects in early primexine formation and later collapsed pollen grains (Paxson-Sowders et al., 2001). DEX1 is expressed in microspores and encodes a predicted integral membrane protein. Curiously, the sterility defect followed Mendelian inheritance, suggesting control by the maternal sporophyte (Paxson-Sowders et al., 2001). Our genetic studies show that loss of male fertility in chx17chx18chx19 mutants is largely a male gametophyte defect and is accompanied by exine deformation. While we cannot eliminate CHX function in tapetal or pollen mother cells, our SEM results (not shown) support the hypothesis that primexine synthesis is initiated from microspores. It is likely that microspores within a tetrad produce a scaffold or template upon which exine materials attach and assemble (Ariiuzumi and Toriyama, 2011). Thus, any defects in the scaffold could directly affect subsequent exine patterning. Alternatively, a defect in intine (or inner wall) that is formed later by bicellular pollen between the PM and the outer wall might perturb exine patterning. These ideas warrant reconsideration.

How could three CHX transporters with nearly identical activities perturb male fertility? To date, our results are consistent with the hypothesis that chx mutants have perturbed membrane trafficking that remodels the PM and the cell wall (Kim and Brandizzi, 2016), and those changes lead to impaired fertility. First, CHX17 as well as CHX18 or CHX19 confer tolerance to alkaline pH in a yeast mutant sensitive to growth at pH 7.5, suggesting a role in pH homeostasis. Secondly, CHX17 supports growth of K+ uptake mutants in yeast and E. coli, suggesting a role in K+ transport and K+ homeostasis. Thirdly, CHX17 could reduce the secretion of vacuolar carboxypeptidase Y in yeast, indicating that it has a role in proper sorting of cargo (Chanroj et al., 2011). As CHX17 has been localized to the PVC and the PM in plant cells (Chanroj et al., 2013), the results infer that proper regulation of the endomembrane pH and cation level plays a role in protein and cargo sorting (Chanroj et al., 2011). This idea is demonstrated convincingly in a det3 H+-pumping V-ATPase
Our results show that chx17chx18chx19 mutant pollen is compromised at multiple phases in reproduction. As many ovules received a pollen tube, we suggest that failed fertilization is mainly due to reduced tube rupture or impaired sperm activity, or both. Loss of function of PM-localized CHX19 could affect tube growth and rupture perhaps through ANX1/ANX2 receptor-like kinases (Boisson-Dernier et al., 2009). Loss of CHX18 and CHX17 function in sperm cells could impair male gamete activity. CHX17 was shown to alter sorting of protein cargo in yeast (Chanroj et al., 2011), thus plasma membrane proteins (e.g. receptors) or secreted factors of the pollen tube or sperm might be misguided in the triple mutant.

HAP2 is a sperm cell-expressed PM protein that is crucial for fertilization (Mori et al., 2006; von Besser et al., 2006). HAP2 is a transmembrane protein that is sorted to the PM from the ER after activation by EC1, an egg cell-secreted peptide that promotes fertilization (Sprunck et al., 2012). A homolog in Chlamydomonas is needed for cell–cell fusion, suggesting that the plant HAP2/GCS1 might act as a gamete fusogen (Liu et al., 2008). GEX2 encodes a PM-localized protein in sperm cells. Mutant sperm cells failed to attach to the egg membrane, indicating that GEX2 is required for gamete–gamete attachment, a prerequisite for successful fertilization (Mori et al., 2014). Perhaps sperm CHX18 influences the sorting and delivery of proteins, such as GEX2 or HAP2 (GCS1), to promote gamete attachment and fusion.

Our findings support a model in which three related cation/H⁺ exchangers affect a subset of endomembranes in developing pollen, pollen tube, sperm, and central cells. Pollen development, tube sensing, or wall properties might be sufficiently compromised to reduce sperm release, sperm function, or both in chx17chx18chx19 mutants. Our study highlights the critical roles of cation/H⁺ exchangers in membrane trafficking, cargo sorting, and wall remodeling for successful fertilization.

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Author contributions

HS conceived the original research plans; HS, AYC, MAJ supervised the experiments; SP, DC, and RS performed most experiments; KL, TKM, ARL, and YZ performed other experiments; SC generated the mutants; SP, DC, ARL, and HS designed the experiments and analyzed the data; HS wrote the article with contributions of all the authors; and AYC and MAJ complemented the writing.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Single T-DNA insertion mutants used in this study.

Table S2. Gene-specific primers and T-DNA border primers used for genotyping plants.

Fig. S1. Flower development of the chx17/chx18/chx19 mutant and the wild type is similar.

Fig. S2. Pod and seed development at 0–9 days after pollination.

Fig. S3. Pollen wall architecture is partially disorganized in chx17/chx18 double mutants but not in the single chx17 mutant.

Fig. S4. Time-course of ovule development in wild-type (WT) pistils receiving WT or chx17/chx18/chx19 mutant pollen.

Fig. S5. Pollen tubes of the chx17/chx18/chx19 mutant entered ovules that remain undeveloped.

Fig. S6. CHX19–RFP was localized to the PM along the flanks of the pollen tube.

Fig. S7. RNA sequencing revealed expression of CHX17 and CHX18 in a laser-dissected central cell.

Fig. S8. Expression of CHX17 and CHX18 in the micropylar and peripheral endosperm during early seed development.

References


